

Isoimmunization against human chorionic gonadotropin with conjugates of processed β -subunit of the hormone and tetanus toxoid

(kinetics of immune response/immunoreactivity of antibodies/mammals/sub-human primates/neutralization of hormonal biological activity *in vitro* and *in vivo*)

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ABSTRACT The immunogenicity of the conjugate prepared from "processed" β -subunit of human chorionic gonadotropin (choriogonadotropin, HCG) and tetanus toxoid has been studied in animals and a human subject. The conjugate elicited the formation of high-affinity ($K_a = 10^9$ - 10^{11} M $^{-1}$) anti-HCG and anti-tetanus antibodies. On primary immunization, the antibody response lasted for several months. Repeat injection of the conjugate in the declining phase of antibody titers produced a booster response without a lag period. The antibodies reacted with the β -subunit of HCG and the complete HCG molecule but were devoid of significant crossreactivity with human growth hormone, placental lactogen, follicle-stimulating hormone, thyroid-stimulating hormone, and luteinizing hormone at tonic and surge levels. The antibodies were competent for neutralizing the biological activity of HCG in the mouse uterine weight gain assay, the ventral prostate weight gain assay, and the radioligand assay for binding of 125 I-labeled HCG to receptors on corpus luteum. HCG (5000 international units) administered to an immunized subject was completely bound by circulating antibodies. Administration of HCG (in contrast to conjugate) was without booster effect on anti-HCG titers.

Human chorionic gonadotropin (choriogonadotropin) (HCG) is a product of trophoblasts and is synthesized at a very early stage of pregnancy, i.e., within 6-8 days of the fertilization of the egg (1). It provides a critical support to the corpus luteum, which decays in its absence, resulting in menstruation. The hormone has a crucial role in establishment and maintenance of pregnancy. HCG is not normally produced by nonpregnant females; the only exceptions are patients with choriocarcinomas (2), hydatidiform mole (3), and patients with cancers that ectopically synthesize the hormone (4, 5). Its early appearance and obligatory role in pregnancy make it a suitable target for control of fertility. Since HCG has to travel through the blood stream to reach the ovarian corpus luteum it is susceptible to inactivation by circulating antibodies.

Active immunization against HCG in humans poses, however, two major problems. First is its expected crossreactivity with other hormones (6) since the α -subunit of HCG is nearly identical to the α -subunits of thyroid-stimulating hormone (thyrotropin), follicle-stimulating hormone (folliotropin), and luteinizing hormone (lutropin) (7-9). The hormone-specific β -subunit (β -HCG) or a moiety thereof would have to be used in such a way that the antiserum against it would be able to react immunologically with the complete HCG molecule, but be devoid of reactivity against other hormones and body tissues. Second, the antiserum should

neutralize the biological activity of HCG. This consideration is important since the antiserum against a tryptic COOH-terminal fragment of HCG, although reacting immunologically with HCG, is not able to form biologically inactive complexes with HCG (10).

HCG, being a "self" protein, would have to be either tagged with haptenic groups or conjugated with immunogenic carriers to be rendered antigenic in humans. The characteristics of the sulfanilic acid derivative of HCG (11) and dinitrophenyl (Dnp) derivatives of β -HCG have been described (12). We report here observations on the immunological properties and biological potential of a conjugate of β -HCG and tetanus toxoid. Some aspects of this work have been presented elsewhere (13-15).

MATERIALS AND METHODS

The Antigen. We purified and "processed" β -HCG by a procedure described elsewhere (16) to obtain a product with minimal crossreactivity with human luteinizing and other hormones. The processed β -HCG (Pr- β -HCG) was conjugated with purified tetanus toxoid (TT) (>1500 limit flocculation units/mg of protein nitrogen) in discrete molecular proportions. The conjugate, Pr- β -HCG-TT, was extensively dialyzed and passed through a Millipore filter (pore size 0.22 μ m). The lack of microbial contamination and pyrogens was tested as described (17).

Antibodies. Anti-HCG titers were determined by radioimmunoassays (18) and anti-TT, by passive hemagglutination as described (19). The ability of the antibodies to neutralize the biological activity was determined by the mouse uterine weight gain assay, the ventral prostate weight gain assay, and the radioligand assay for binding of 125 I-labeled HCG to receptors on corpus luteum (20).

RESULTS

Antibody Response to Pr- β -HCG-TT. This conjugate was found to be antigenic in mice, rabbits, a goat, and monkeys. Fig. 1 shows the results of a representative experiment in a goat and monkeys. The antibodies appeared after a lag period and continued to rise for several months, after which they reached a plateau. In monkeys there was a concomitant rise in anti-TT antibody titers. In the goat, however, a second injection was required before antibodies to TT were produced.

Elsewhere (19), we have shown that the antibody titers declined in course of time in primates; thus, the antibody response to this conjugate antigen was reversible. We have also shown elsewhere (19) that a repeat injection of the conjugate

Abbreviations: HCG, human chorionic gonadotropin; β -HCG, β -subunit of HCG; TT, tetanus toxoid; Pr- β -HCG-TT, conjugate of processed β -subunit of human chorionic gonadotropin and tetanus toxoid; IU, international unit; HA, hemagglutination.

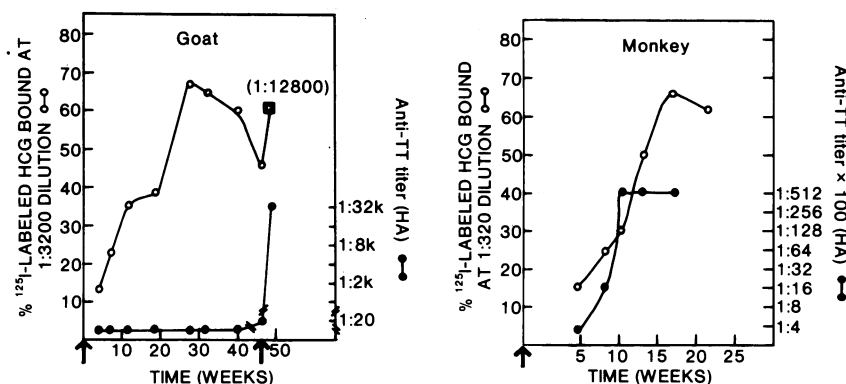


FIG. 1. Kinetics of formation of anti-HCG and anti-TT antibodies in a goat and rhesus monkeys by Pr- β -HCG-TT. Arrows indicate day of injection of the conjugate. Anti-TT titers were determined by hemagglutination (HA) and $k = 10^3$.

gave a booster response and that the affinity constants (K_a) of the antibodies for binding with HCG were of the order of 10^9 – 10^{11} M $^{-1}$ and showed a tendency to increase with time.

Immunological Reactivity. The antibodies produced by this conjugate reacted immunologically not only with β -HCG but also with the complete native hormone. They were devoid of crossreactivity with a number of other human hormones, e.g., growth hormone (somatotropin), placental lactogen, follicle-stimulating hormone, thyroid-stimulating hormone, and luteinizing hormone at tonic and surge levels (Table 1).

Biological Activity. Since large polymeric antigens give rise to the production of heterogeneous antibodies reacting with different determinants on the molecule, it is conceivable that the antibodies produced by this conjugate react with determinants other than those implicit in the biological activity of the hormone. The ability of the antibodies generated by this conjugate to neutralize the biological activity of HCG was thus tested in four different experimental systems (20). Typical results obtained in two of the *in vivo* and one

in vitro procedure are given in Table 2 and Figs. 2 and 3. The antiserum was effective in abrogating the binding of HCG with corpus luteum receptors and the bio-response of target organs to HCG.

Toxicology. The conjugate fulfils pharmacopeial safety requirements as defined in our ref. 17. Acute toxicity studies indicated Pr- β -HCG-TT up to a dose of 200 limit flocculation units/kg intramuscularly in mice was safe. The conjugate was without any significant effects on gross behavior and central nervous systems of mice and rats. Intravenous administration (80–200 μ g/kg) of the conjugate in anesthetized cats increased uterine contraction without any significant effect on blood pressure or respiration. Lower doses or intramuscular administration produced no change in these parameters (21). When several times the human equivalent doses was given to 115 mice, 6 rabbits, and 19 monkeys, no death due to the conjugate was noted up to periods beyond 1 year (22). The conjugate is thus well tolerated in a number of animal species. The chronic toxicity has been studied in monkeys for periods beyond 1 year, during which anti-HCG

Table 1. Specificity of anti Pr- β -HCG-TT sera from monkey and goat

Competing hormone*	Quantity in reaction mixture (ng)	% 125 I-labeled HCG bound to antibody, $B/B_0 \times 100$		% competition with 125 I-labeled HCG $100 - [(B/B_0) \times 100]$		Quantity at which significant competition appears (ng) [†]
		Monkey (1:16,000)	Goat (1:12,800)	Monkey	Goat	
β -HCG (CR-115)	1.0	30	42	70	58	0.15
HCG (CR-115)	1.0	49	57	51	43	0.5
Human luteinizing hormone (LER-960)	1.2 §	100	96	0	4	5
	0.4 ‡	100	98	0	2	—
Human follicle-stimulating hormone	0.7 §	100	100	0	0	50
(LER-1575-C)	0.3 ‡	100	100	0	0	—
Human thyroid-stimulating hormone (Pierce Fraction 4)	2.5 §	100	100	0	0	100
Human placental lactogen (Potency estimate 95%)	12.5 §	100	100	0	0	>1000
Human growth hormone (NIH-GH HS 1652-C)	0.25 §	100	100	0	0	>1000

* Obtained from the U.S. National Institutes of Health.

[†] A competition, $100 - [(B/B_0) \times 100]$, of 20% or above was considered significant. B = radioactive material (cpm) bound in presence of competing hormone; B_0 = radioactive material (cpm) bound in presence of labeled hormone alone.

[‡] Basic or tonic concentration, and § maximum physiological concentration, calculated on the basis of the potency of the individual hormone used and the reported values (29).

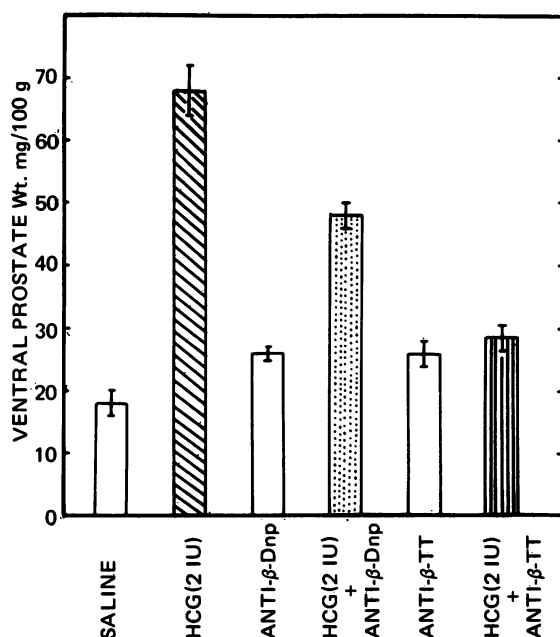


FIG. 2. Effect of anti Pr-β-HCG-TT and anti β-HCG-Dnp sera on the HCG-induced gain in ventral prostate weight. Pr-β-HCG is abbreviated to β.

antibodies were maintained in the animal at reasonably high levels. The immunized monkeys maintained good health, and gained weight. No abnormality in metabolic, endocrine, and organ functions was noticeable in these monkeys (23). Post-mortem examination of four immunized monkeys after 7–17 months of high antibody titers showed no immunological or toxic damage in the endocrine and reproductive organs. Immunocomplex nephritis was not seen in the kidneys. All organs examined were grossly and microscopically normal.

Hypersensitivity and Reactivity with other Tissues. Sera from three human subjects immunized with Pr-β-HCG-TT vaccine were tested for possible reactivity with other human tissues. These were found to be negative for antinuclear and antimicrosomal antibodies, and rheumatoid factor. The sera from these subjects did not give any reaction with human thyroid, parathyroid, adrenal, testes, ovaries, and fetal and adult pituitaries, as detected by immunofluorescence techniques, nor did they react with tissue substrates obtained from baboons, mice, and rabbits (24).

In nine monkeys immunized with this conjugate, immediate Arthus and delayed-type hypersensitivity skin reactions to intradermal HCG did not occur (25).

Immunogenicity in Human Subjects. Phase I clinical trials in a limited number of human subjects were started with this conjugate in March–April, 1974. All subjects im-

Table 2. Effect of monkey anti-Pr-β-HCG-TT on mouse uterine weight

Injection	Mean uterine weight in mg (95% confidence limits)
Saline	4.67 (3.49–5.85)
Anti-Pr-β-HCG-TT	4.86 (2.40–7.31)
HCG (0.3 IU)	12.33 (9.89–14.78)
HCG + Anti-Pr-β-HCG-TT	4.95 (4.31–5.59)

IU, international unit.

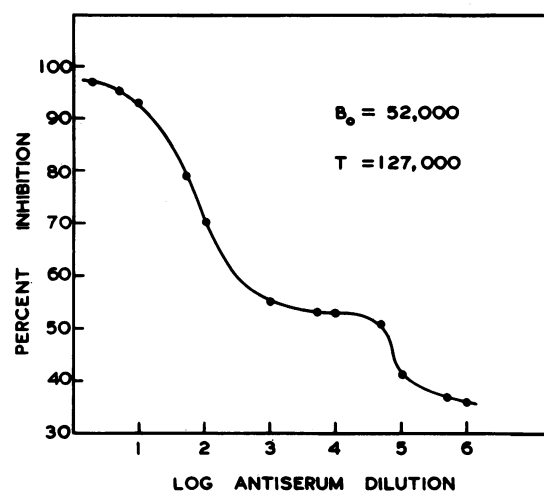


FIG. 3. Antiserum generated in monkeys by injection of Pr-β-HCG-TT inhibits binding of 125 I-labeled HCG to goat corpora lutea. B_0 denotes cpm bound to receptors and T the total cpm added.

munized with the conjugate responded positively with the formation of anti-tetanus and anti-HCG antibodies. K.W. (Fig. 4) is a representative subject. After a lag period of 4–6 weeks, her antibodies increased progressively and attained plateau levels around 4 months. The plateau was maintained for about 3 months, after which a slow decline was noticed. The subject (K.W.) who was 31 years old had had 4 pregnancies and 1 abortion, and a hysterectomy and tubal ligation on March 12, 1974. She was immunized with the conjugate on April 3, 1974 and continued to have regular menstrual cycles throughout the period of study. Endometrium biopsy (Fig. 5) and progesterone levels which ranged from 7.85 to 9.58 ng/ml in blood samples taken in the secretory phase of the cycle at different stages of the study indicated that she continued to ovulate. The antibodies formed blocked the binding of 125 I-labeled HCG to receptors in the corpus luteum (20).

HCG Clearance. It was pertinent to enquire whether the antibodies generated by the conjugate in human subjects had the right affinity and were present in adequate amounts

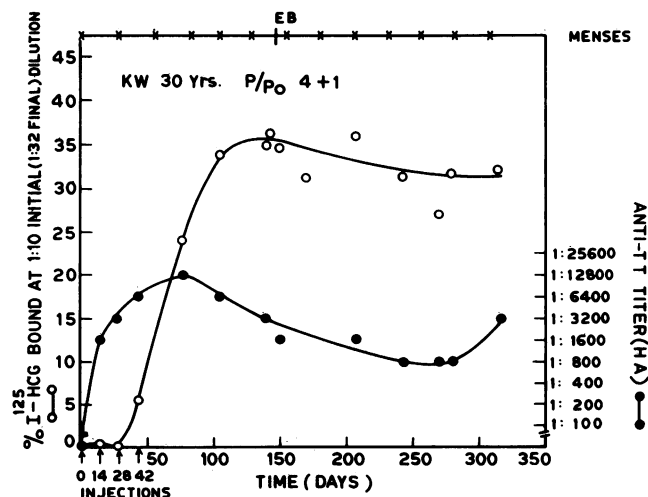


FIG. 4. Kinetics of anti-HCG and anti-TT titers in a human subject (K.W.) injected with Pr-β-HCG-TT. Xs at the upper abscissa indicate the dates of menstruation and EB the date at which endometrial biopsy was done.

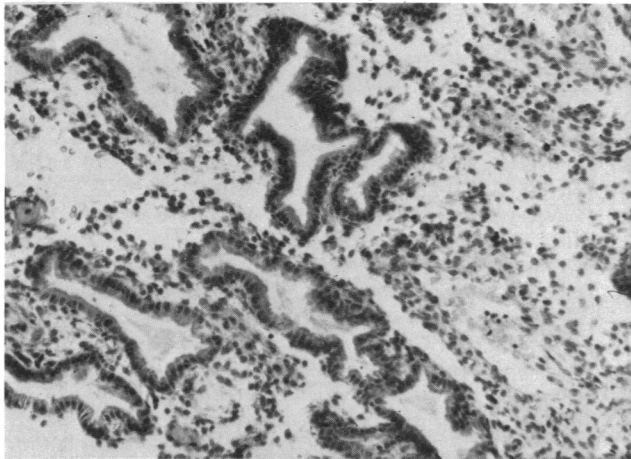


FIG. 5. Endometrial biopsy of K.W. (cycle 10) in premenstrual phase. The micrograph shows the secretory endometrium ($\times 160$).

to take care of HCG produced in the event of a pregnancy. HCG (5000 IU) was injected intramuscularly into the subject and blood samples were drawn at frequent intervals. Fig. 6 shows a steep fall in titer of circulating anti-HCG, indicating the utilization of the antibodies by the administered HCG. The antibody titers stayed at low levels (but above zero) for 72 hr, after which they started rising and in time returned to the original levels. Since HCG load did not alter the circulating anti-TT antibody titers in this subject, independence of reactivity of anti-HCG and anti-tetanus antibodies is suggested. Similar results have been obtained on another subject immunized with Pr- β -HCG-TT (27), in whom the antibodies present in circulation 11 months after vaccination were adequate to bind 2000 and 4000 IU of HCG given at a 24-hr interval.

Clinical Pharmacology. This subject and others injected with the conjugate have been followed at regular intervals for nearly 13 months. They are completely normal clinically and careful investigations of metabolic, endocrine, reproductive, and other organ functions have not revealed any abnormality (28).

DISCUSSION

A "self" protein can be made immunogenic either by tagging it with powerful haptenic groups or by conjugating it with an immunogenic protein carrier. The choice of tetanus toxoid as a carrier for the present purpose was based on a number of considerations: (i) it is one of the purest bacterial antigens available and has a very low incidence of local reactions, discomfort, and fever; (ii) it evokes immunity of a duration which is advantageous for health care programmes; (iii) it is approved for human use; and (iv) tetanus is an appreciable health hazard, especially in developing countries. The present studies show that Pr- β -HCG-TT elicits antibodies not only to HCG but also against tetanus. It has thus a double benefit.

Although the diazotized sulfanilic acid derivatives of HCG (11) or Dnp derivatives of β -HCG (12) elicit the formation of anti-HCG antibodies, the TT conjugates may have merit not only because of their additional utility as protective agents against tetanus but also because they are antigenic at much lower doses than are hapten-conjugated antigens. The neutralizing ability of the antisera raised with TT-conjugated material is also superior to that of antisera raised with β -HCG-Dnp.

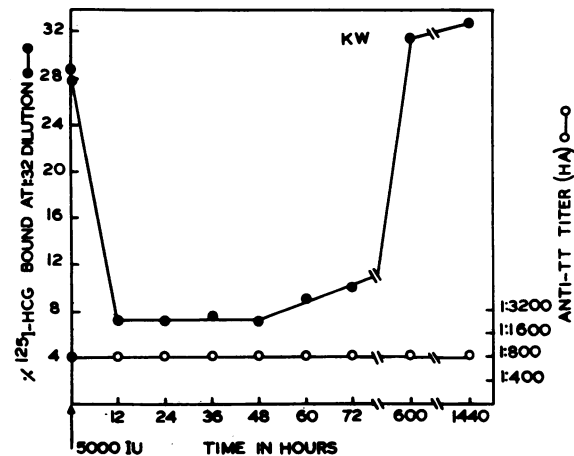


FIG. 6. Effect of administration of HCG (5000 IU) on K.W. immunized with Pr- β -HCG-TT. The anti-HCG and anti-TT titers have been measured in blood samples drawn at several time periods after HCG load. Arrow indicates the time of injection (zero hour).

Pr- β -HCG-TT is immunogenic in many mammals, including the rhesus monkeys, and in human subjects. The conjugate has the right conformation to expose the antigenic determinants on the polymer that are either directly implicit in the biological activity of the hormone or whose combination with the antibodies leads to an inactive conformation of the HCG molecule. Examples are known of hormonal polypeptides such as β -subunit of thyroid-stimulating hormone (9) and the COOH-terminal fragment of β -HCG (10) in which the antigenic determinants and those involved in biological activity are not identical.

The duration of the antibody response with this conjugate is reasonably long in animals (19) and in humans (26). In some animals and in one of the human subjects immunized with Pr- β -HCG-TT, the antibody titers fell to nearly zero level after 1 year. In others, however, the antibodies were present more than 15 months after injection. Thus, the average duration of primary immune response to this conjugate in humans is expected to be around 1 year.

Since the antibody concentrations decline almost to those of nonimmunized controls, the antibody response to primary immunization with Pr- β -HCG-TT is reversible in both monkeys and humans. A second injection of the vaccine in the declining phase of the antibodies produces a marked booster response, without a lag period.

The antibodies generated by this conjugate are fairly specific. *In vitro*, they are devoid of crossreactivity with human placental lactogen, growth hormone, follicle-stimulating hormone, thyroid-stimulating hormone, and luteinizing hormone at the tonic and surge levels. This specificity is maintained in the human subjects. No disturbance in menstrual cycle or in lactation (28) was seen in subjects immunized with the conjugate. Evidence for continued ovulation in immunized subjects was obtained by endometrium biopsy, vaginal cytology, and serum progesterone levels in the secretory phase of the cycle.

Antibodies produced by this conjugate were free from reactivity with other human organs, e.g., pituitary, thyroid, parathyroid, adrenals, testes, and ovaries, as tested by immunofluorescence. No antinuclear, antimicrobial antibodies or rheumatoid factor type of reactivities were detected in sera from either the immunized animals or the human subjects. Immunization with the conjugate did not produce

immediate Arthus or delayed-type hypersensitivity to intradermal challenge with HCG in monkeys (25) or immunized human subjects.

Immunization does not apparently produce any disturbance in kidney, liver, adrenal, thyroid, cardiovascular, and reproductive organ functions (23, 28).

HCG injections into immunized human subjects show that the antibodies generated by this conjugate bind HCG *in vivo*. Furthermore, the circulating antibodies in the immunized subjects, even at modest titers, are adequate to bind 5000 IU of injected HCG (subject reported in this paper) and 2000 and 4000 IU of injected HCG (another subject reported elsewhere, ref. 27).

HCG production is assumed to start from zero and increase progressively with proliferation of trophoblasts. In an immunized subject, the HCG produced initially would encounter a situation of antibody excess. Not only would this ensure an effective neutralization of the hormone, but also the antigen-antibody complexes in conditions of antibody excess would not pose the problem of immune complex deposits usually encountered in conditions of antigen excess.

Another deduction permissible from these observations (Fig. 6) is that HCG alone does not produce a secondary booster response in subjects immunized with Pr- β -HCG-TT. If boosting had occurred, pregnancy would reinforce the anti-HCG immune response and possibly block the occurrence of pregnancy at a later date. The entire conjugate has to be injected to induce a booster response.

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